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FEMS Microbiology Letters 190 (2000) 309–316

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Recombinant BCG approach for development of vaccines: cloning and expression of immunodominant antigens of *M. tuberculosis*

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Received 3 May 2000; received in revised form 13 July 2000; accepted 13 July 2000

Abstract

In spite of major advances in our understanding of the biology and immunology of tuberculosis, the incidence of the disease has not reduced in most parts of the world. In an attempt to improve the protective efficacy of *Mycobacterium bovis* bacille Calmette–Guérin (BCG), we have developed a generic vector system, pSD5, for expression of genes at varying levels in mycobacteria. In this study, we have cloned and overexpressed three immunodominant secretory antigens of *M. tuberculosis*, 85A, 85B and 85C, belonging to the antigen 85 complex. All the genes were cloned under the control of a battery of mycobacterial promoters of varying strength. The expression was analysed in the fast-growing strain *M. smegmatis* and the slow-growing vaccine strain *M. bovis* BCG. The recombinant BCG constructs were able to express the antigens at high levels and the majority of the expressed antigens was secreted into the medium. These results show that by using this strategy the recombinant BCG approach can be successfully used for the development of candidate vaccines against infections associated with mycobacteria as well as other pathogens.

Keywords: Mycobacterium; Gene expression; Recombinant bacille Calmette–Guérin; Antigen 85 complex

1. Introduction

Tuberculosis (TB) remains a major health challenge worldwide. More than 50 000 deaths every week, about 8 million new cases of TB every year [1,2] and the emergence of drug-resistant strains of *M. tuberculosis* have emphasised once again that drug therapy has its intrinsic limitations. Bacille Calmette–Guérin (BCG), the only vaccine available against TB, has provided consistent protection against the childhood form of TB, especially meningitis [3]. However, it has a history of controversy due to variations in its efficacy against the adult TB observed in different studies and populations [4]. Thus, a vaccine that can provide consistent protection against adult TB is badly needed. The challenge of the disease fortunately has been matched by new technologies becoming available during the last two decades. Efforts have been concentrated upon subunit, auxotrophic, atypical, recombinant BCG, and DNA vaccines [5–10]. However, the excellent immunomodulating properties of BCG, its proven safety for human use and the failure of alternative candidate vaccines to improve upon the performance of BCG have led to the concept of recombinant BCG-based vaccines against TB and other infections.

We have earlier reported the construction of a generic vector system, pSD5, for mycobacteria [11] which facilitates cloning of promoters as well as genes in a modular fashion thus making it feasible to express an antigen at different levels for elicitation of optimal immune response. In the present study, the 85 complex antigens [12,13] were cloned in the pSD5 expression system under transcriptional control of a battery of mycobacterial promoters of varying strength. This complex comprises three closely related components, antigen 85A, 85B and 85C which together constitute 20–30% of all proteins present in the supernatant of short-term cultures [13] and thus represent one of the most promising groups of extracellular proteins as vaccine candidates. These antigens were expressed in the absence and presence of their secretory signals. The expression was analysed in the fast-growing mycobacterial species *Mycobacterium smegmatis* as well as in the slow-growing vaccine strain *M. bovis* BCG to exploit the usefulness of this strategy for the development of vaccines against TB and other infectious diseases.

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PII: S0378-1097(00)00353-0

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1.

2.2. Bacterial culture and transformation

Escherichia coli DH5α, *M. smegmatis* LR222 and *M. bovis* BCG were cultured and transformed as described earlier [14].

2.3. Cloning of various mycobacterial promoters into pSD5

A mycobacterial promoter library in the promoter probe vector pSD7 was constructed in our laboratory previously [15]. Two oligonucleotides, PrP1 and CAT (Table 1), were synthesised for amplification of the promoter fragments cloned upstream of the chloramphenicol acetyltransferase (CAT) reporter gene in pSD7. The location and orientation of these primers is depicted in Fig. 1. pSD7 constructs carrying promoters of varying strengths were used as templates and amplification reaction was carried out by using Pfu DNA polymerase (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The PCR amplicons were end-repaired, phosphorylated and cloned in the blunted *Xba*I site of pSD5 vector to generate pSD5.pro constructs. Recombinants were screened for correct orientation by restriction analysis.

2.4. PCR amplification and cloning of the candidate genes

Oligonucleotides were designed from the sequences of genes for the 85 antigen complex in the databank (accession numbers: X53898 for 85A, X62398 and M21839 for 85B and X57229 for 85C). A strategy was designed for the amplification of the full-length gene and for amplification of the portion encoding only the mature protein (devoid of the signal peptide). Keeping the downstream primer common, two primers were designed for the upstream region, one primer annealing at the N-terminal region and amplifying the full-length gene (W) and the other primer annealing after the region encoding the signal peptide and amplifying the portion encoding only the mature protein (T). The schematic location of primers and the expected amplified products are depicted in Fig. 2A. The pair of primers used for the amplification of the full-length 85A gene were 5′ primer 85AI and 3′ primer 85AII. The pair of primers used for the amplification of the region encoding the mature 85A protein were 5′ primer 85AIII and 3′ primer 85AII. A similar strategy was employed for the amplification of genes for 85B and 85C. The primer sequences are shown in Table 1. All the 5′ primers carried an *Nde*I site and all the 3′ primers carried an *Mlu*I site in

Table 1
Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant information	Source or reference
Strains		
E. coli DH5α	*supE*44 Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	[15]
M. smegmatis LR222	High-frequency transforming strain	[15]
M. bovis BCG (Glaxo)	Vaccine strain	[14]
M. tuberculosis H_{37}Rv	Virulent strain of mycobacteria originally isolated from a TB patient	Dr Jaya Tyagi, AIIMS, New Delhi, India
Plasmids		
pZERO-2.1	Cloning vector, Amp^r, ccdB peptide	Invitrogen, Carlsbad, CA, USA
pSD7	Mycobacterial promoter probe vector, kn^r, oriM, p15A	[15]
pSD5	*E. coli*–mycobacteria shuttle vector, kn^r, oriM, p15A	[11]
pSD7.pro	Promoter derivative of pSD7	[15]
pSD5.pro	Promoter derivative of pSD5. Promoter cloned in *Xba*I site	This work
Primers[a]		
PrP1	5′-GAGTGCTTCCAGCACGGGCG-3′	[14]
CAT	5′-CCTGAAAATCTCGTCGAAGCTCGGCGG-3′	[14]
85AI	5′-GGGAAGCA**catATG**CAGCTTGTTGACAGGGTTCG-3′	This work
85AII	5′-aaatagtac**tacgcgt**CTAGGCGCCCTGGGGCGCGGGCCCGG-3′	This work
85AIII	5′-aagatatc**atatg**TTTTCCCGGCCGGGCTTGCCGGTGG-3′	This work
85BI	5′-GGGGCACA**caTATG**ACAGACGTGAGCCGAAAGATTCG-3′	This work
85BII	5′-gccagtac**tacgcgt**CCCGTCAGCCGGCGCCTAACGAACTCTGC-3′	This work
85BIII	5′-aagatatc**atatG**TTCTCCCGGCCGGGGCTGCCGG-3′	This work
85CI	5′-GGTAGTA**caTATG**ACGTTCTTCGAACAGGTGCG-3′	This work
85CII	5′-aaatagtac**tacgcgt**TCAGGCGGCCGGCGCAGCAGGGGCGGCCGG-3′	This work
85CIII	5′-aagatatc**atatg**TTCTCTAGGCCCGGTCTTCCAGTGG-3′	This work

[a]In primer sequences, lowercase letters denote nucleotides added or modified to facilitate incorporation of restriction sites marked in bold.

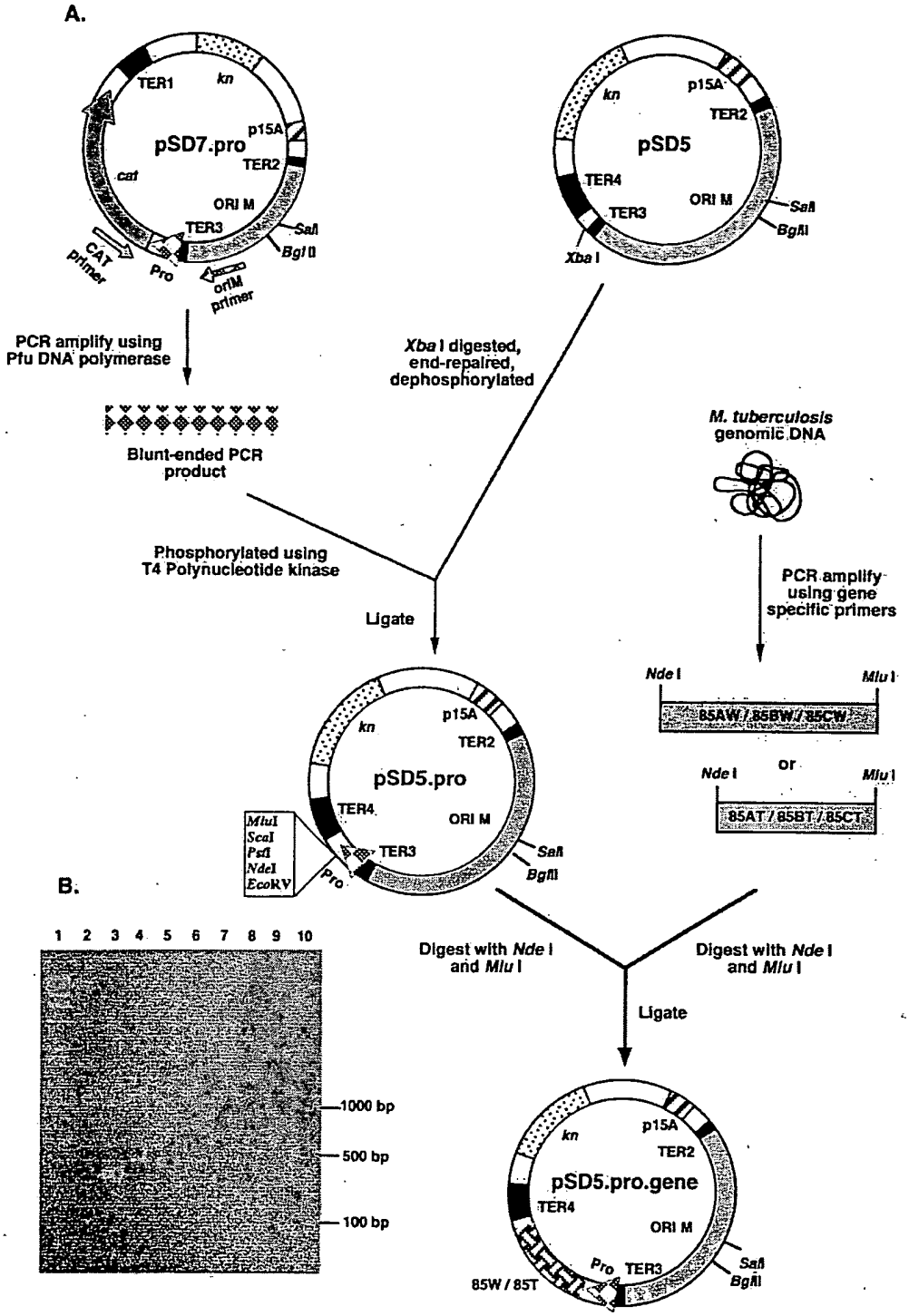

Fig. 1. A: Construction of the pSD5.pro gene construct. Details of the construction are described in the text. Relevant restriction sites and antibiotic resistance marker (kn, kanamycin) are shown. The transcriptional terminators are designated TER2, TER3 and TER4, which represent the rrnBT1, the synthetic tryptophan terminator of *E. coli* and the *E. coli* fd terminators respectively. ORI M represents the mycobacterial origin of replication. B: Ethidium bromide-stained 1.2% agarose gel showing amplification of some mycobacterial promoters from the mycobacterial promoter library. The amplification reaction was carried out as described in the text. Lane 1: λ-*Hin*dIII marker; lane 2: control reaction with all components of the PCR reaction except the template; lanes 3–9: amplification reactions of different promoters using constructs pSD7.T106, pSD7.T31, pSD7.S35, pSD7.S16, pSD7.αcry respectively as the template; lane 10: 100-bp DNA ladder.

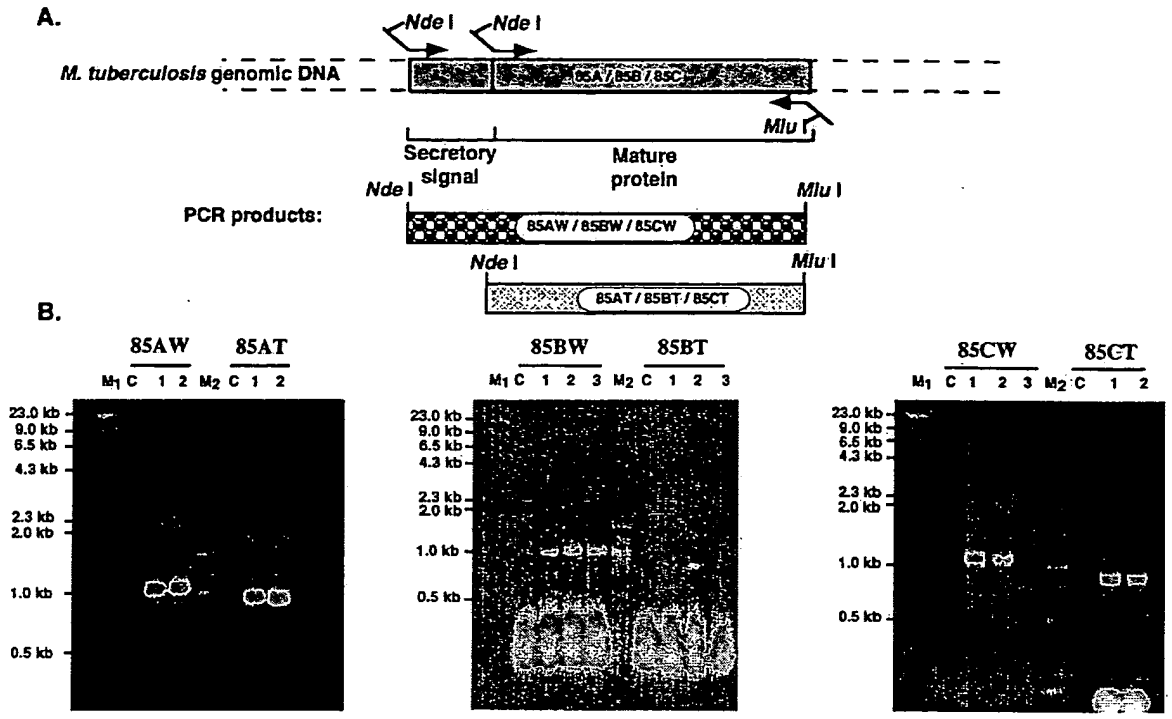

Fig. 2. PCR amplification of the genes for the candidate antigens. A: Schematic illustration of the location of different primers used for the PCR reaction, in the context of the gene and the PCR products expected (full-length gene represented as W and the truncated gene, devoid of the region encoding the signal peptide, designated T). B: Agarose gel electrophoresis of the PCR products using *M. tuberculosis* H37Rv genomic DNA as the template. The PCR reaction was carried out as described in the text. Scale at left indicates the size of the molecular mass markers. Label at top of the gel indicates the amplified gene. Suffix W indicates the full-length gene, including the portion encoding the secretory signal and suffix T indicates the portion encoding only the mature protein. Lane M_1: λ-*Hin*dIII DNA molecular mass marker; lane M_2: 100-bp DNA ladder; lane C: control reaction with all components of the PCR reaction except the template; lanes 1–3: amplification reaction of different genes at varying magnesium concentrations (1.5, 2.0 and 3.0 mM respectively) using *M. tuberculosis* H_{37}Rv genomic DNA as the template.

the overhangs (shown in bold in the primer sequences) for directional cloning of these amplification products into pSD5. The PCR reaction was carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA), using Pfu DNA polymerase (Stratagene, La Jolla, CA, USA) according to the manufacturer's guidelines. The PCR-amplified products were electrophoresed on a 1% agarose gel and stained with ethidium bromide (Fig. 2B). The amplified products were processed as described above and cloned into the *Eco*RV site of vector pZERO-2.1 (Invitrogen, San Diego, CA, USA). Sequencing of the recombinant clones using the SP6 and M13 primers confirmed the cloning of correct target sequences. The PCR products were taken out as *Nde*I–*Mlu*I fragments and cloned directionally into *Nde*I–*Mlu*I-digested pSD5 and its promoter derivatives, described previously (Fig. 1).

2.5. Expression of the full-length and truncated 85 complex antigens in M. smegmatis LR222

The plasmids containing the genes for antigens 85A, 85B and 85C under different promoters were propagated in *E. coli*, purified and then electroporated into *M. smegmatis* LR222 as described earlier [14]. Individual colonies from LB agar plates containing kanamycin (25 μg ml $^{-1}$) were inoculated into 6 ml of LB medium containing 0.5% glycerol, 0.2% Tween-80 and 25 μg ml $^{-1}$ of kanamycin. The cultures were grown for 2–3 days with shaking (200 rpm) at 37°C after which the cells were pelleted at 6000×*g* for 10 min. All the cultures were harvested at an OD_{600} of 2.0–3.0. The supernatant was collected and protein precipitated with five volumes of ice-cold acetone. The cells were resuspended in phosphate-buffered saline (PBS) and sonicated on ice for 1 min with 15-s pulses and 15-s intervals between pulses using a probe sonicator. The sonicate was pelleted at 8000×*g* and supernatant collected. Protein in the culture supernatant and cell-free extracts was estimated using Bradford's reagent [16]. Equal amounts of protein (30 μg) were subjected to 0.1% SDS–10% PAGE according to Laemmli [17] and stained with 0.25% Coomassie brilliant blue R250. Protein expression was analysed in *M. smegmatis* LR222 carrying the plasmids harbouring the genes for the 85 complex antigens.

2.6. Expression of the candidate antigens in M. bovis BCG

The constructs containing the genes for antigens 85A, 85B and 85C under different promoters were electroporated into the vaccine strain *M. bovis* BCG as described earlier [14]. BCG transformants were grown to mid-expo-

nential phase in 7H9 medium containing 10% volume of ADC enrichment (Difco, Detroit, MI, USA) and kanamycin (25 μg ml $^{-1}$). Liquid culture medium included 0.5% glycerol and 0.2% Tween-80. Cultures were harvested at OD_{600} of 2.0–3.0 and the samples were processed as described for *M. smegmatis* LR222 except that sonication was carried out for a period of 3 min.

2.7. Immunoblotting

Protein samples were electrophoresed on a 0.1% SDS–10% PAGE and then transferred to nitrocellulose paper (pore size 0.45 mm) at 40 mA overnight or at 180 mA for 2 h using the Bio-Rad Mini Trans-Blot Cell (Bio-Rad, Hercules, CA, USA). Non-specific binding sites on the membrane were blocked with 1% bovine serum albumin for 1 h before the membrane was incubated overnight with HYT 27, a monoclonal antibody against 85 complex antigens (WHO-IMMTUB mAb bank, courtesy of Dr. T. Shinnick, Centers for Disease Control and Prevention, Atlanta, GA, USA) at a dilution of 1:100. After washing the blot with PBS containing 0.05% Tween-20 the blot was incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulin G at a dilution of 1:2500. The blots were then washed and immunoreactive bands visualised by the addition of 0.1% of 30% H_2O_2 and 0.5 mg ml $^{-1}$ 3,3′-diaminobenzidine tetrahydrochloride.

3. Results

3.1. Cloning of various mycobacterial promoters into pSD5

We chose five promoters for the present study – T106, T31, S16, αcry (from the mycobacterial promoter library constructed in our laboratory previously) [15] and hsp (*M. leprae* hsp65) [18], a kind gift of Dr Douglas B. Young, Department of Infectious Diseases and Microbiology, Imperial College School of Medicine, London, UK. The strength of these promoters ranged from 500 to 18000 nmol min $^{-1}$ mg $^{-1}$ of CAT activity. These promoters were PCR-amplified (Fig. 1B) and cloned in pSD5 in the

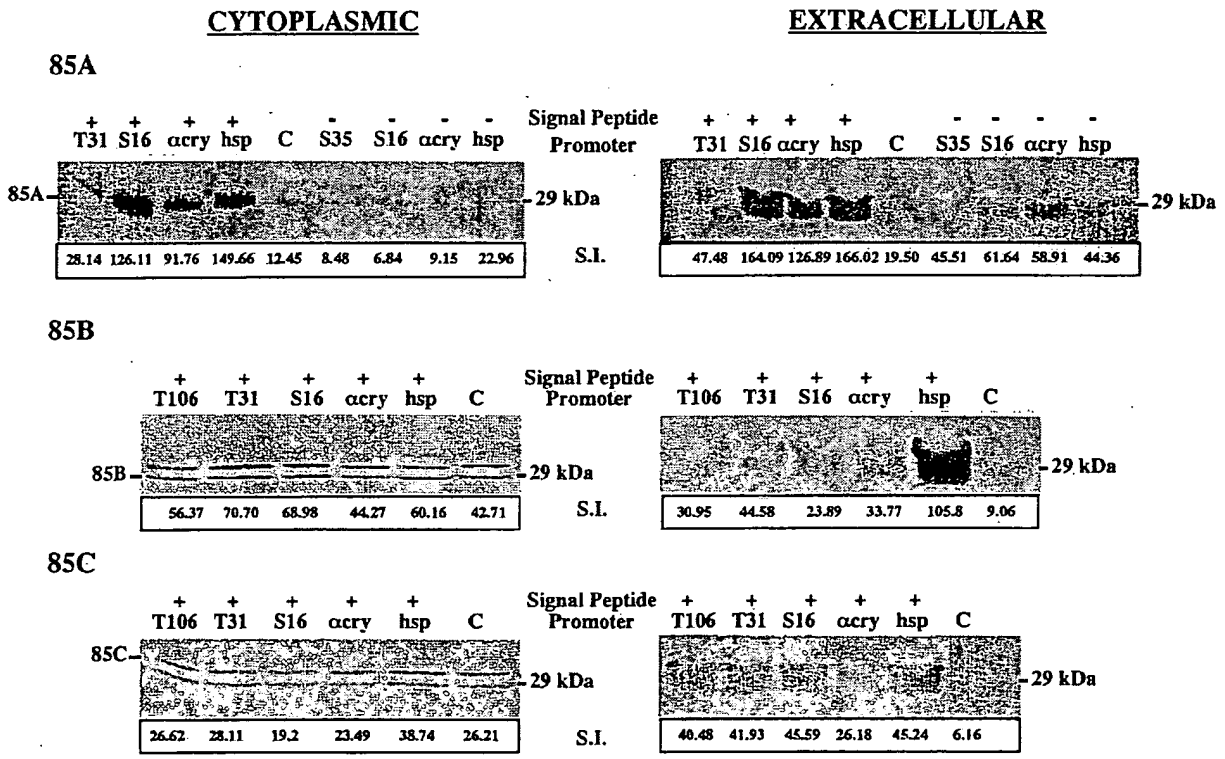

Fig. 3. Analysis of the expression of 85 complex antigens in *M. smegmatis* LR222 by Western blot analysis. Cell-free extracts and culture supernatant of *M. smegmatis* LR222 transformed with various pSD5.pro gene constructs were analysed on a 10% SDS–polyacrylamide gel. Details of the culture conditions and preparation of cell-free extract and culture supernatant are described in the text. Equal amounts of protein (30 μg) were loaded in each lane. The proteins were transferred to nitrocellulose membrane and the blot was probed with antibody to Ag85. Lanes marked T106, T31, S16, αcry, hsp refer to extracts of recombinant *M. smegmatis* LR222 carrying the constructs with these promoters upstream of the gene for 85A or 85B or 85C in pSD5.pro gene. C represents the control lane with extracts of wild-type *M. smegmatis* LR222 transformed with pSD5 carrying the gene without any promoter. The panel on the left represents the cell-free extracts and the panel on the right represents the culture supernatants. The position of the molecular mass marker is indicated. + or − represents the presence or absence of the signal peptide upstream of the cloned gene. S.I. represents the signal intensity in arbitrary units. The value shown below each lane represents the intensity of the band and was calculated using the NIH Image software ver. 1.52.

promoter cloning site, *Xba*I (Fig. 1A). The modular expression cassette of pSD5 has three different compartments, namely (a) cloning sites for promoters, (b) ribosome binding sites along with an ATG codon and (c) multiple cloning sites for cloning the genes to be expressed. The first two compartments are separated by translational termination codons in all three reading frames to uncouple transcription from translational initiation [11]. The cloning of the promoters was confirmed by restriction analysis.

3.2. PCR amplification and cloning of candidate genes

The genes encoding the antigen 85 complex antigens were PCR-amplified with signal peptide (represented as W) and without single peptide (represented as T). For this, the downstream primer was kept common and two primers were designed for the 5′ region, one which along with the downstream primer would amplify the full-length gene and the other that would amplify only the portion encoding the mature protein. Fig. 2B shows the amplification of the genes. The amplicons were digested with *Nde*I–*Mlu*I and cloned directionally in pSD5.pro downstream of various promoter fragments, as shown in Fig. 1A. Cloning in the *Nde*I site ensured the correct reading frame without addition of any extra amino acids from the vector sequences as the *Nde*I site itself contains the translational start site, ATG.

3.3. Expression of the candidate antigens in M. smegmatis LR222

We have observed earlier that *M. tuberculosis*, *M. bovis*, and *M. smegmatis* recognise mycobacterial promoters with similar efficiencies, and the transcriptional machineries of these species share common determinants of transcriptional specificity [14]. Hence, in a preliminary analysis of our constructs, we analysed the expression of the cloned genes in the fast-growing saprophyte *M. smegmatis* LR222. Expression of the antigens was monitored by immunoblot-

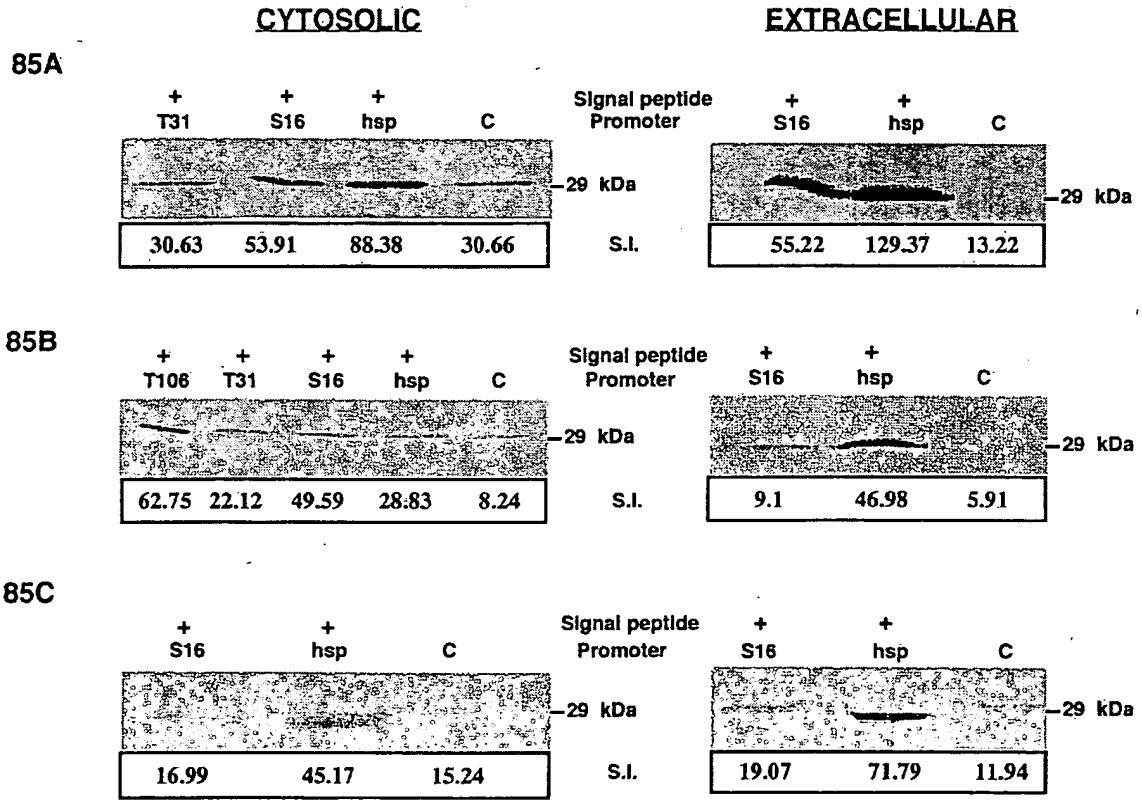

Fig. 4. Analysis of the expression of 85 complex antigens in *M. bovis* BCG by Western blot analysis. Cell-free extracts and culture supernatant of *M. bovis* BCG transformed with various pSD5.pro gene constructs were analysed on a 10% SDS–polyacrylamide gel. Equal amounts of protein (30 μg) were loaded in each lane. The proteins were transferred to nitrocellulose membrane and the blot was probed with antibody to Ag85. Lanes marked T106, T31, S16, αcry, hsp refer to extracts of recombinant *M. bovis* BCG carrying the constructs with these promoters upstream of the gene for 85A or 85B or 85C in pSD5.pro gene. C represents the control lane with extracts of wild-type *M. bovis* BCG transformed with pSD5 carrying the gene without any promoter. The panel on the left represents the cell-free extracts and the panel on the right represents the culture supernatants. Scale at right indicates the position of molecular mass markers. + or represents the presence or absence of the signal peptide upstream of the cloned gene. S.I. represents the signal intensity in arbitrary units. The value shown below each lane represents the intensity of the band and was calculated using the NIH Image software ver. 1.52.

ting (Fig. 3). The monoclonal antibody HYT27 recognises all the three antigens (85A–31 kDa, 85B–30 kDa, 85C–31.5 kDa), therefore, a 30/32-kDa doublet is observed in immunoblots [19,20]. Since all three antigens are highly secretory antigens, the expression was analysed both in the cell-free extract and in the culture supernatant. The size of the recombinant protein corresponded to the native protein and the expression of the antigens from the recombinant *M. smegmatis* strain was much higher than the native level of expression from *M. smegmatis*. The immunoblots were scanned and the intensity of the signals calculated using the NIH Image software ver. 1.52 (NIH Image by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA. Anonymous FTP: zippy.nimh.nih.gov). As seen in Fig. 3 the expression in the culture supernatants of the recombinants was 2–12-fold higher than that of the control. However, in cytosolic fractions the enhancement was observed only in the case of 85A (2–12-fold). Recombinants of the other two antigens (85B and 85C) showed very little increase (1.1–1.65-fold) in the cytosolic fraction over the native level of expression. The hsp promoter supported maximum expression in the case of all three antigens. In the case of transformants where the genes were amplified along with the region encoding the signal peptide, the majority of the recombinant expressed protein was found in the culture supernatant. Surprisingly, the constructs that were devoid of the secretory signal did not show significant expression (data shown only for 85A, Fig. 3). We had expected increased expression of these antigens from these constructs in the cytosol. However, no significant enhancement was observed in the expression of these antigens from these constructs either in the cytosol or in the supernatant. This suggests that in the absence of signal peptides these proteins are either inherently unstable or more susceptible to some proteolytic degradation.

3.4. Expression of the candidate antigens in *M. bovis* BCG

After confirming the expression of the various antigens in *M. smegmatis* LR222, we analysed expression of all three antigens in the slow-growing vaccine strain *M. bovis* BCG. The constructs were electroporated into *M. bovis* BCG and transformants selected on 7H10 plates containing kanamycin. Expression of the recombinant antigens was analysed by immunoblotting using the monoclonal HYT27 (Fig. 4). As in the case of *M. smegmatis*, expression of the antigen proteins was observed over and above the native level of expression seen in *M. bovis* BCG. In this case also the proteins were secreted efficiently into the extracellular milieu and increased (2–10-fold) amounts of the antigens (85A/85B/85C) were detected in the culture supernatant compared to the levels secreted by native BCG. Moreover, scanning of these blots revealed that the fold increase of expression of these antigens was more in the culture supernatant compared to the cytosolic fraction, as was seen in *M. smegmatis*. Immunoblots of some of these constructs are shown in Fig. 4. A single band was observed in native *M. bovis* BCG as well as in the recombinant BCG extracts. As mentioned before a doublet was observed in *M. smegmatis* in the case of both native and recombinant strains. This suggests some difference in the pattern of expression of these proteins in the two hosts. These proteins, however, lack post-translational modification [21]. Hence, the observed difference in the migration of antigen proteins is not associated with glycosylation or lipoacylation.

4. Discussion

BCG vaccine holds the paradoxical position of simultaneously being the most widely used as well as the most controversial vaccine today [4,22]. The failure of other candidate vaccines against TB in animal studies has led to the proposal that the existing BCG vaccine should be modified by overexpression of promising antigens to develop a more potent vaccine against TB. An important dictate from studies using the rBCG approach states that for optimal exploitation of this approach, different antigens will have to be expressed at different levels for elicitation of optimal immune response in the case of every antigen [23]. Most previous studies using the rBCG approach for expression of viral and bacterial antigens were limited by the choice of mycobacterial expression signals and have employed the hsp65 promoter. However, the expression obtained using this promoter did not always result in an optimal immune activation. Hence, these studies suffered from the lack of an expression system that would allow a range of expression levels for any antigen. In a systematic approach towards this goal, we have developed a vector system pSD5 [11] for expression of various genes in *M. bovis* BCG at different levels. In this study, we have cloned and expressed the genes for all three 85 complex antigens of *M. tuberculosis* in BCG using this expression system.

M. bovis BCG represents an attractive tool as a delivery vehicle for the development of rBCG vaccines. In this study, we demonstrate that employing the vector system pSD5 and various mycobacterial promoters, the strategy described here can be used for expressing any antigen at varying levels in BCG. The ideal vaccine candidate among these can then be selected by screening based on their evaluation in animal models. In principle any candidate foreign gene can be introduced and expressed in BCG at various levels using the system described in this report. This strategy thus makes it feasible to express any antigen in BCG to induce elicitation of optimal immune response. This will significantly extend the scope of the rBCG approach for the development of vaccines against other infections. Recent studies have demonstrated that rBCG expressing protective viral or bacterial antigens may induce strong cellular immune responses and under some circum-

stances good antibody responses. Thus, the rBCG approach with the strategy described here represents an attractive candidate for the development of live recombinant vaccines against infections of mycobacteria and other pathogens.

Acknowledgements

We thank J.S. Tyagi for critical reading of the manuscript. This work was carried out through a grant from the Department of Biotechnology, Government of India. N.D. and V.R. are thankful to the University Grants Commission, India for Senior Research Fellowships. The technical assistance of Bindu Nair and Manisha Jain is acknowledged. Rajiv Chawla is acknowledged for efficient secretarial assistance.

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